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ORIGINAL PAPER

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Nicotinic acid controls lactate production by K1-LDH: a *Saccharomyces cerevisiae* strain expressing a bacterial LDH gene

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Abstract Industrial applications for lactate, such as the production of chemicals, has led to interest in producing this organic acid by metabolically engineered a yeast such as *Saccharomyces cerevisiae*, which is more acid tolerant than lactic acid bacteria. This paper deals with lactate production by *S. cerevisiae* K1-LDH, in which the *Lactobacillus plantarum* lactate dehydrogenase (LDH) gene is integrated into the genome of the wine yeast strain K1. We show that a vitamin, nicotinic acid (NiA), was the limiting factor for lactate production during fermentation with the K1-LDH strain. Increasing the NiA concentration in batch conditions or in the medium used to feed chemostats affected the lactate yield. Moreover, the addition of pulses of NiA or the exponential addition of NiA made it possible to control the lactate production kinetics throughout the fermentation process. The results point to the role of NiA in the regulation of metabolic pathways, but the physiological mechanisms remain poorly understood.

Keywords Nicotinic acid · L-lactate · Alcoholic fermentation · Lactate dehydrogenase · *Saccharomyces cerevisiae*

Introduction

Lactic acid is used for an increasing number of applications. For example, it is used as a monomer in biodegradable polymer(s), which provides the potential to greatly expand the market for this product. To make the price of lactic acid more attractive, the efficiency and economics of lactic acid production need to be improved. Industrial lactic acid production processes use lactic acid bacteria. These conventional processes have two main disadvantages: (1) the low pH (due to lactate production) inhibits the bacterial metabolism and (2) expensive purification procedures are often required (e.g., chemical extraction). Thus, there is growing interest in using alternative methods to produce lactic acid. These methods include fermentation processes with microorganisms that are able to grow and to metabolize at very low pH. Yeasts such as *Saccharomyces cerevisiae* tolerate low pH, but only produce small amounts of lactate during conventional fermentation [14, 19].

The way in which pyruvate (generated during glycolysis) is converted through the final electron acceptor system for NAD regeneration differs between yeast and lactic acid bacteria. In yeast, pyruvate decarboxylase converts pyruvate into acetaldehyde and CO_2; and alcohol dehydrogenase then reduces the acetaldehyde to ethanol. In lactic acid bacteria, pyruvate is converted into lactate by lactate dehydrogenase (LDH).

Some authors [1, 7, 13, 17] recently showed that metabolically engineered *S. cerevisiae* strains expressing a LDH gene could be used to shift the glycolytic flux towards the production of lactate. In this study, we used an engineered yeast strain carrying the LDH gene from *Lactobacillus plantarum* and showed the effect of fermentation conditions on lactate production. After demonstrating the importance of vitamins, we focused on the effect of nicotinic acid (NiA), which was found to be the main factor controlling lactate production.

Materials and methods

Yeast culture

Yeast strains

We used the lactate-producing, genetically modified yeast strain K1-LDH, which is the K1 (ICV-INRA) commercial wine *S. cerevisiae* yeast strain carrying the LDH gene from *L. plantarum*. The LDH gene was

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integrated into the genome of K1. Construction details are given by Colombié et al. [6].

Medium

Fermentation experiments were carried out in two defined media: synthetic medium (SM) and nitrogen-free medium (YNB). SM (composition described by Bely et al. [3]) contained 200 g l^{-1} glucose, acids (DL-malic acid, citric acid) and five groups of elements: (1) nitrogenous compounds (0.42 g l^{-1} of assimilable nitrogen as a mixture of ammoniac and amino acids), (2) salts (KH_2PO_4, K_2SO_4, $MgSO_4$, $CaCl_2$, NaCl), (3) trace elements [$MnSO_4$, $ZnSO_4$, $CuSO_4$, KI, $CoCl_2$, H_3BO_3, $(NH_4)_6Mo_7O_{24}$], (4) anaerobic factors (ergosterol, oleic acid, Tween 80) and (5) vitamins (thiamine, biotin, pyridoxine, meso-inositol, pantothenic acid, NiA). YNB medium (1.7 g l^{-1} bacto yeast nitrogen base without amino acids and ammonium sulfate, DIFCO, COGER) was supplemented with 1.98 g l^{-1} ammonium sulfate (0.42 g l^{-1} of assimilable nitrogen) and 200 g l^{-1} glucose. It was buffered in the same way as SM with 6 g l^{-1} DL-malic acid and 6 g l^{-1} citric acid (final pH 3.60) and supplemented with one of these five groups: nitrogenous compounds, salts, trace elements, anaerobic factors or vitamins, depending on the experiment.

Culture conditions

YPD medium (100 ml; 1% bacto yeast extract, 2% bactopeptone, 2% glucose) was inoculated with cells from an agar plate. The culture was incubated for 24 h at 28°C with shaking at 300 rpm and then transferred to a reactor (10^9 cells l^{-1}). The culture broth had previously been sterilized in steam (100°C) for 10 min. The bioreactors were equipped with air locks and stirred with magnetic stirrers. Fermentation experiments were carried out at 28°C.

Batch cultures were run in 0.3-l or 1.2-l non-commercial bioreactors. In the 1.2-l bioreactors, the pH was monitored with an on-line pH sensor (Mettler HA 405) and was maintained at pH 3.60 by the addition of 10 M KOH. The total volume of base added did not exceed 3% of the fermentation volume.

For chemostat cultures, a 0.35-l working volume was kept constant via an effluent line coupled to a peristaltic pump. Cultures were assumed to be in a steady state when: (1) at least six volume changes had occurred since the last change in growth conditions and (2) the culture did not exhibit metabolic oscillations.

Nicotinic acid was added manually (pulses) or using a computer-controlled pump (exponential addition). CO_2 release was automatically measured from the mass lost every 20 min (about 100 g of CO_2 is released during the entire fermentation process). The rate of CO_2 production ($dCO_2/d\,t$, where t is time) was calculated by polynomial smoothing of the last ten values of CO_2 production. This technique was validated for the estimation of sugar and alcohol concentrations in fermentation experiments with wild-type wine yeast strains (such as K1) [16]. In the case of K1-LDH, the correlation between the amount of lactate produced and the amount of KOH added for pH regulation [6] enabled us to estimate the precise lactate concentration and the precise lactate production rate. Residual sugar and alcohol concentrations were determined from the amount of CO_2 and lactate produced, with an error of less than 5%. All fermentation experiments were run in duplicate or triplicate.

Analytical methods

Biomass

The number of yeast cells was determined using an electronic particle counter (Coulter ZM2, Electronics) equipped with a 100-μm orifice.

Products

L-Lactate and glycerol concentrations were determined by HPLC on a HPX-87H Aminex column (Biorad), using H_2SO_4 (0.008 N) as the mobile phase with a flow rate of 0.6 ml min^{-1}. Dual detection was performed with a refractometer and an UV detector (at wavelength 210 nm; Hewlett Packard 5890). Glucose concentration was determined by use of the dinitrosalicylic reagent [11].

Results

Nicotinic acid: a key vitamin

The level of lactate produced was about eight-fold higher (and the yeast population at the stationary phase about three-fold larger) following fermentation on SM than on YNB medium (Table 1, lines 1, 2, 8, 9). To determine which of the nutrients in SM are involved in lactate production, each class of compounds (nitrogen sources, salts, trace elements, anaerobic factors, vitamins) was added individually to YNB medium. Lactate production and K1-LDH growth were both dramatically increased when YNB medium was supplemented with vitamin solution (Table 1, lines 2, 7, 9, 10). As reported by Colombié et al. [6], two lactate production yields were observed: a low yield during the growth phase (i.e., during one-third of the fermentation) and a higher yield during the stationary phase. Interestingly, the addition of vitamins to YNB medium increased lactate production both during the growth phase (increasing the first yield $y_{lact/S1}$ from ca. 0 to 0.09 ± 0.005 g g^{-1}) and during the stationary phase (increasing the second yield $y_{lact/S2}$ from 0.05 ± 0.003 g g^{-1} to 0.16 ± 0.008 g g^{-1}; Fig. 1).

To determine whether only one vitamin (among the six in the mixture) is required, fermentation experiments were performed omitting each vitamin individually (i.e., with YNB medium supplemented with five of the

Table 1 Effect of medium composition, pH regulation and vitamins, especially NiA, on lactate production. Fermentation experiments (lines 1–7) were performed in a 250-ml reactor without pH regulation; and all other fermentation experiments were performed in a 1.2-l reactor with pH regulated at 3.6. *X* Number of cells

Line	Fermentation medium	Initial NiA ($mg\ l^{-1}$)	Final lactate ($g\ l^{-1}$)	Final glucose ($g\ l^{-1}$)	X (10^9 cells l^{-1})
1	SM	2.0	26.1 ± 1.3	< 2.0 ± 0.1	200 ± 10
2	YNB	0.4	3.4 ± 0.2	< 2.0 ± 0.1	60 ± 3
3	YNB + nitrogenous nutrients[a]	0.4	2.3 ± 0.1	< 2.0 ± 0.1	60 ± 3
4	YNB + salts[a]	0.4	0.9 ± 0.1	< 2.0 ± 0.1	60 ± 3
5	YNB + trace elements[a]	0.4	2.8 ± 0.1	9.5 ± 0.5	60 ± 3
6	YNB + anaerobic factors[a]	0.4	1.7 ± 0.1	< 2.0 ± 0.1	75 ± 4
7	YNB + vitamins[a]	2.4	9.7 ± 0.5	7.6 ± 0.4	110 ± 6
8	SM	2.0	42.9 ± 2.2	3.0 ± 0.2	180 ± 9
9	YNB	0.4	5.2 ± 0.3	< 2.0 ± 0.1	70 ± 4
10	YNB + vitamins[a]	2.4	27.0 ± 1.4	< 2.0 ± 0.1	120 ± 6
11	YNB + vitamins[a] without thiamine	2.4	28.5 ± 1.4	< 2.0 ± 0.1	115 ± 6
12	YNB + vitamins[a] without biotin	2.4	24.9 ± 1.3	< 2.0 ± 0.1	130 ± 7
13	YNB + vitamins[a] without NiA	0.4	10.9 ± 0.6	9.2 ± 0.5	120 ± 6

[a]The amount added corresponded to the concentration in the SM

six vitamins). The omission of NiA reduced the lactate yield by about 2.5-fold, by decreasing both of the lactate production yields: $y_{lact/S1}$ from 0.09 ± 0.005 g g^{-1} to 0.03 ± 0.002 g g^{-1} and $y_{lact/S2}$ from 0.16 ± 0.008 g g^{-1} to 0.09 ± 0.005 g g^{-1} (Fig. 1, Table 1, lines 10–13). The omission of NiA did not significantly change the duration of fermentation (about 110 h) or the yeast population at the stationary phase. Conversely, the omission of thiamine or biotin (the main vitamins involved during growth) did not noticeably affect lactate production (Table 1, lines 10–13).

Whatever the medium (SM, YNB ± vitamins), fermentation experiments performed without pH regulation (0.3-l reactor) and with pH regulation at 3.6 (1.2-l reactor) showed that lactate production was enhanced by up to 2.5-fold when the pH was maintained at 3.6, although the yeast population at the stationary phase was not affected (Table 1, lines 1, 2, 7–10). Therefore, to precisely identify the effect of NiA on K1-LDH fermentation, all subsequent fermentation experiments were performed at pH 3.6 in SM medium. Indeed, even with a higher initial concentration of NiA (2.4 mg l^{-1} instead of 2.0 mg l^{-1} in SM medium), less lactate was produced with vitamin-supplemented YNB medium than with SM medium (Table 1, lines 1, 7, 8, 10).

We performed two sets of experiments: (1) with increasing initial concentrations of NiA in batch medium or in continuous fermentation feed medium and (2) adding NiA (pulses or exponential addition) during batch fermentation.

Effect of NiA concentration

Batch experiments

Batch fermentation experiments were performed with different initial concentrations of NiA in SM medium. In the absence of NiA: (1) the yeast population was very small in the stationary phase (55×10^9 cells l^{-1}), (2) less than half of the sugar was consumed after 300 h and (3) the lactate production was negligible (Tables 2, 3, 4). This indicates that NiA is required for growth.

The maximum glucose consumption rate $(dS/dt)_{max}$ was lower for the lowest initial concentration of NiA (0.4 mg l^{-1}) than for the other concentrations (Table 3). In the same way, the final concentration of glycerol was

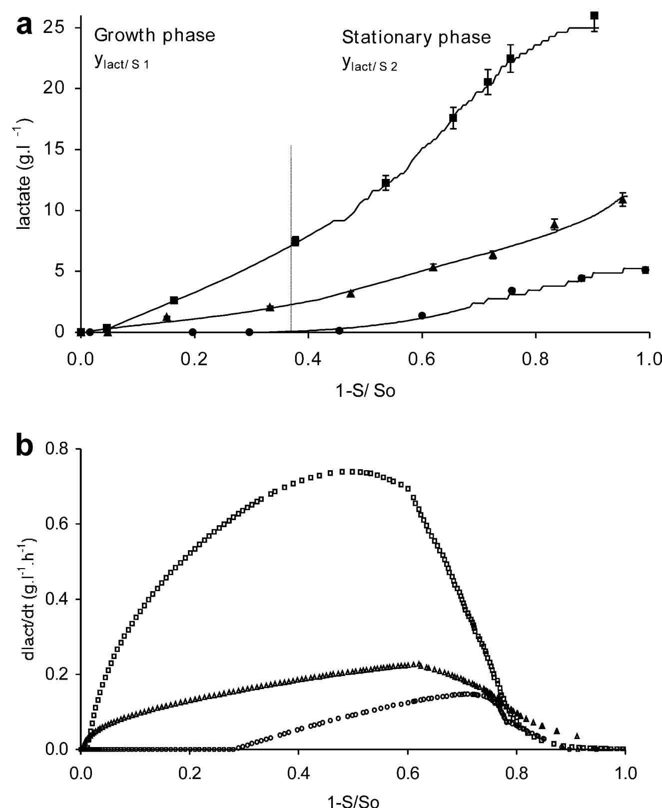

Fig. 1a, b Effect of vitamins on lactate production kinetics. **a** Off-line measurements (*symbols*) and on-line estimates (*solid lines*) of lactate production with the two lactate production yields $y_{lact/S1}$ and $y_{lact/S2}$. **b** Lactate production rate for fermentation experiments carried out in YNB medium with pH regulated at 3.6 (*circles*), in YNB supplemented with vitamin solution (*squares*) and in the same medium without NiA (*triangles*)

Table 2 Effect of initial NiA concentration on K1-LDH lactate production in experiments carried out in SM with pH regulated at 3.6. $Y_{lact/S}$ Apparent lactate yield from glucose calculated for the whole fermentation. *P* Lactate productivity calculated for the whole fermentation [in sluggish fermentation experiments, the final time is estimated when the glucose consumption rate (dS/d*t*) < 0.1 g l^{-1} h^{-1}]

Initial NiA (mg l^{-1})	Final lactate (g l^{-1})	$(dlact/dt)_{max}$ (g l^{-1} h^{-1})	$y_{lact/S,1}$ (g g^{-1})	$y_{lact/S,2}$ (g g^{-1})	$Y_{lact/S}$ (g g^{-1})	*P* (g l^{-1} h^{-1})
0.0	0.9 ± 0.1	~0	~0	~0	~0	
0.4	16.6 ± 0.8	0.3 ± 0.02	0.04 ± 0.01	0.12 ± 0.01	0.08 ± 0.01	0.21 ± 0.02
1.2	36.9 ± 1.9	1.0 ± 0.05	0.12 ± 0.01	0.26 ± 0.03	0.19 ± 0.02	0.37 ± 0.04
2.0	43.6 ± 2.2	1.3 ± 0.07	0.12 ± 0.01	0.32 ± 0.03	0.22 ± 0.02	0.44 ± 0.04
4.0	50.2 ± 2.5	1.4 ± 0.07	0.18 ± 0.02	0.37 ± 0.04	0.26 ± 0.03	0.48 ± 0.05
10.0	39.8 ± 2.0	1.4 ± 0.07	0.17 ± 0.02	0.32 ± 0.03	0.20 ± 0.02	0.27 ± 0.03

Table 3 Effect of initial NiA concentration on K1-LDH fermentation characteristics in experiments carried out in SM with pH regulated at 3.60

Initial NiA (mg l^{-1})	$(dS/dt)_{max}$ (g l^{-1} h^{-1})	X (10^9 cells l^{-1})	Final glucose (g l^{-1})	Duration (h)	Glycerol (g l^{-1})
0.0	1.4 ± 0.1	55 ± 3	112.5 ± 5.6	> 300	
0.4	5.4 ± 0.3	190 ± 10	< 2.0 ± 0.1	80 ± 4	7.5 ± 0.4
1.2	6.9 ± 0.4	190 ± 10	< 2.0 ± 0.1	100 ± 5	6.0 ± 0.3
2.0	6.8 ± 0.3	190 ± 10	2.8 ± 0.1	100 ± 5	6.3 ± 0.3
4.0	6.2 ± 0.3	190 ± 10	4.0 ± 0.2	95 ± 5	6.3 ± 0.3
10.0	6.2 ± 0.3	190 ± 10	< 2.0 ± 0.1	128 ± 6	6.3 ± 0.3

affected by the initial NiA concentration at the lowest concentration (0.4 mg l^{-1}).

Increasing the initial NiA concentrations from 0.4 mg l^{-1} to 4.0 mg l^{-1} increased the final lactate concentration, the maximum lactate production rate $(dlact/dt)_{max}$ and the lactate production yields during the growth and stationary phases (Table 2). The highest lactate production was observed with 4.0 mg l^{-1} of NiA. When the NiA concentration was further increased to 10 mg l^{-1}, the lactate production properties (kinetics, yield) remained constant or tended to decrease.

Increasing the initial NiA concentration from 0.4 mg l^{-1} to 10.0 mg l^{-1} did not affect the yeast population in the stationary phase (Table 3). All of the fermentations occurred correctly with less than 4.0 ± 0.2 g l^{-1} residual sugar. More surprisingly, the duration of the fermentation increased with the highest initial concentration of NiA.

Continuous culture (chemostat)

To determine whether the NiA concentration affects lactate production during the growth phase, we performed continuous culture experiments with increasing NiA concentrations in the medium feed (SM). The dilution rate, $D = 0.05$ h^{-1} was chosen according to our previous results [6], as it allows maximum sugar consumption.

Increasing the NiA concentration from 0.4 mg l^{-1} to 4.0 mg l^{-1} increased the lactate production, but the maximum amount of lactate produced was 15.0 ± 0.8 g l^{-1} (Table 5). With the highest NiA concentration (6.0 mg l^{-1}), the lactate production kinetics decreased slightly. The yeast population and the steady-state glucose consumption were not significantly affected by the NiA concentration, at least in the range 0.4–6.0 mg l^{-1} (Table 5). In the batch experiments, the best yield and productivity were achieved for NiA concentrations of 2.0–4.0 mg l^{-1}. However, growing cells produced low lactate yields ($Y_{lact/S}$) with the same magnitude as the lactate production yield of the batch growth phase ($y_{lact/S1}$, cf. Tables 2, 3, 4).

Effect of adding NiA

NiA (initial concentration in the range 0.4–2.0 mg l^{-1}) controls lactate production. To study this effect further, we carried out fermentation experiments adding NiA in

Table 4 Effect of initial NiA concentration on K1 fermentation characteristics in experiments carried out in SM with pH regulated at 3.6

Initial NiA (mg l^{-1})	$(dS/dt)_{max}$ (g l^{-1} h^{-1})	X (10^9 cells l^{-1})	Final glucose (g l^{-1})	Duration (h)	Glycerol (g l^{-1})
0.0	2.1 ± 0.1	60 ± 3	76.4 ± 3.8	> 300	10.6 ± 0.5
0.4	7.1 ± 0.4	160 ± 8	< 2.0 ± 0.1	92 ± 5	8.1 ± 0.4
1.2	7.1 ± 0.4	190 ± 10	< 2.0 ± 0.1	77 ± 4	6.4 ± 0.3
2.0	6.9 ± 0.4	190 ± 10	< 2.0 ± 0.1	76 ± 4	6.1 ± 0.3
4.0	6.7 ± 0.3	190 ± 10	< 2.0 ± 0.1	72 ± 4	6.4 ± 0.3
10.0	7.1 ± 0.4	190 ± 10	< 2.0 ± 0.1	70 ± 4	6.1 ± 0.3

Table 5 Effect of the initial concentration of NiA on lactate production in continuous fermentation. The experiment was carried out at $D = 0.05\ h^{-1}$ in SM with pH regulated at 3.6

NiA (mg l^{-1})	Lactate (g l^{-1})	Glucose consumed (g l^{-1})	X (10^9 cells l^{-1})	$Y_{lact/S}$ (g g^{-1})	*P* (g l^{-1} h^{-1})
0.4	4.4 ± 0.2	95.0 ± 4.8	110 ± 6	0.05 ± 0.01	0.22 ± 0.01
2.0	15.2 ± 0.8	110.0 ± 5.5	110 ± 6	0.14 ± 0.01	0.76 ± 0.04
4.0	15.0 ± 0.8	100.0 ± 5.0	110 ± 6	0.15 ± 0.02	0.75 ± 0.04
6.0	12.0 ± 0.6	100.0 ± 5.0	110 ± 6	0.12 ± 0.01	0.60 ± 0.03

two different ways: (1) pulses during the stationary phase—with non growing cells—and (2) exponential addition during the whole fermentation period.

Pulses of NiA during the stationary phase

Fermentation experiments were performed with an initial NiA concentration of 0.4 mg l^{-1}, to allow basic growth but low lactate production. We then added NiA (1.6 mg l^{-1}) during the stationary phase. This addition triggered lactate production (Fig. 2). In all conditions, two lactate production yields were observed (Fig. 2a): the first one was low and similar to that observed in batch conditions with an initial concentration of 0.4 mg l^{-1} NiA ($y_{lact/S1} = 0.04 \pm 0.004$ g g^{-1}, Table 2). The second yield, which occurred just after the addition, was higher, similar to the control (2.0 mg l^{-1} NiA, $y_{lact/S2} = 0.32 \pm 0.03$ g g^{-1}). All of the fermentations occurred correctly with less than 2 g l^{-1} residual glucose. The earlier the NiA pulse was added, the higher the maximum lactate production rate [$(\mathrm{dlact}/\mathrm{d}t)_{max} = 1.9 \pm 0.1$, 0.90 ± 0.05 and 0.30 ± 0.02 g l^{-1} h^{-1} for additions at 1-S/So = 0.35, 0.55 and 0.85, respectively; Fig. 2b]. Moreover, when NiA was added at 1-S/So = 0.35, the maximum lactate production rate was higher (1.9 ± 0.1 g l^{-1} h^{-1}) than in the control (1.30 ± 0.07 g l^{-1} h^{-1}).

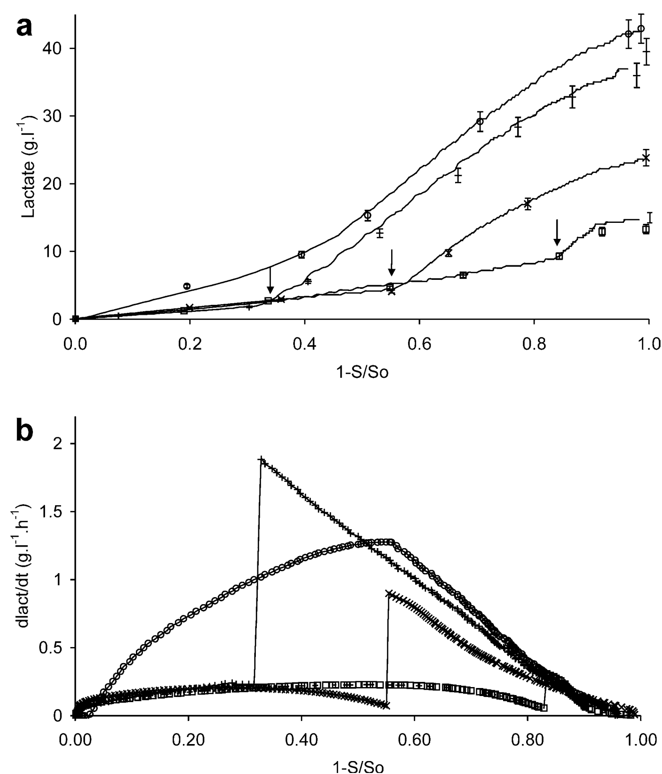

Fig. 2a, b Effect of adding NiA pulses (1.6 mg l^{-1}, indicated by *arrows*) on the kinetics of lactate production. **a** Off-line measurements (*symbols*) and on-line estimates (*solid lines*) of lactate concentration and **b** lactate production rate. Fermentation experiments carried out in SM containing an initial NiA concentration of 0.4 mg l^{-1} (pH regulated at 3.6). NiA was added during the stationary phase at (1-S/So) = 0.35 (*plusses*), 0.55 (*crosses*) and 0.85 (*squares*). The control (*circles*) shows the fermentation in SM (initially 2.0 mg l^{-1} NiA)

Exponential addition of NiA

To determine whether the NiA controls the fermentation process and lactate production, we simulated a progressive addition by adding exponentially 2 mg l^{-1} NiA in 100 h. The kinetics of lactate production were controlled by the NiA addition throughout the entire fermentation period (Fig. 3a). Lactate production stopped when the sugar was exhausted at the end of the fermentation, i.e. when 1.6 mg l^{-1} NiA had been added. The lactate production rate increased as NiA was added (Fig. 3b).

The exponential addition was less effective at triggering lactate production than were pulses: the apparent lactate yield was 0.05 ± 0.01 g g^{-1} and the maximum lactate production rate reached at the end of the fermentation was 0.45 ± 0.02 g l^{-1} h^{-1}.

Discussion

We showed that NiA is the compound that has the greatest effect on lactate production under the experimental conditions evaluated in this study. We previously showed [6] that pH and either glucose or nitrogen concentrations do not affect lactate yield. For example, increasing the initial sugar concentration from 50 g l^{-1} to 280 g l^{-1} led to lactate production yields of $y_{lact/S1} = 0.12 \pm 0.01$ g g^{-1} for the growth phase and $y_{lact/S2} = 0.29 \pm 0.03$ g g^{-1} for the stationary phase.

NiA is required for both growth and lactate production: without NiA, growth was dramatically affected and only traces of lactate were produced. Above a threshold concentration of NiA (between 0.4 mg l^{-1} and 1.2 mg l^{-1}), growth was unchanged and the NiA concentration of the medium controlled lactate production. Interestingly, even though lactate production occurred

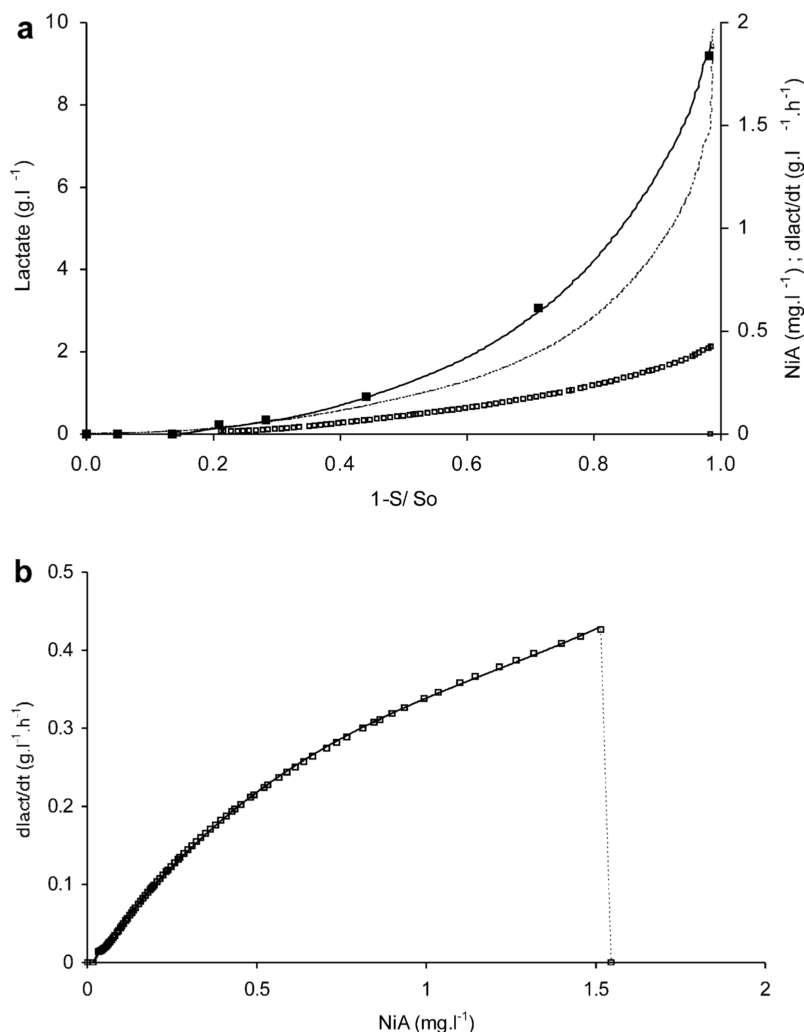

Fig. 3a, b Effect of the exponential addition of NiA on the kinetics of lactate production. **a** Off-line measurements (*filled squares*) and on-line estimates (*solid line*) of lactate concentration, lactate production rate (*open squares*) and amount of NiA added (*dashed line*). **b** Changes in the lactate production rate during the exponential addition of NiA. Fermentation was carried out in SM with pH regulated at 3.6

mostly during the stationary phase, when the NiA concentration was increased from 0.4 mg l^{-1} to 2 mg l^{-1} during fermentation, lactate production was triggered both (1) when cells were growing (chemostat) and (2) when cells were in the stationary phase of the fermentation (batch). Furthermore, lactate production was inhibited by high initial concentrations of NiA (more than 4 mg l^{-1}).

A slightly higher final concentration of glycerol was detected at the lowest initial NiA concentration (Table 3). Low initial NiA concentrations also led to growth limitation and a higher final concentration of glycerol for the wild strain, K1 (Table 4). Thus, NiA—up to a threshold concentration—is limiting for the general yeast metabolism, which is in agreement with yeast NiA auxotrophy under anaerobic conditions [12].

Above 1.2 mg l^{-1} initial NiA, the lactate production by K1-LDH increased, although no significant change was observed for the final concentration of glycerol. The redox balance is not affected and NiA may have an activation effect on LDH activity.

NiA (and nicotinamide, also called niacin) belongs to the B-group of vitamins and is a well known precursor of cofactors NAD+ and NADP+, both of which are required for numerous dehydrogenases, e.g. lactate and malate dehydrogenase. In yeast, the kynurenine pathway from tryptophan generates the nicotinate moiety of NAD if oxygen is available but yeast cells are auxotrophic for NiA under anaerobic conditions [12]. There are reports on the biosynthesis of NiA [2], the kinetics of NiA production by *S. cerevisiae* [18] and the genes involved in NiA biosynthesis [9] and transport [10]. However, the mechanism of how NiA regulates metabolic pathways has never been described.

As the LDH gene from the lactic acid bacterium *L. plantarum* has been introduced into the yeast K1, yielding the K1-LDH strain, this LDH may have specific regulation properties. NiA is an essential vitamin for the growth of a *Lactobacillus* strain [8]. For lactic acid production by *L. casei*, B-vitamins can be used to reduce the amount of yeast extract required (highly effective as an excellent source of B-vitamins, but very expensive) [20]. Nevertheless, NiA cannot be considered as a limiting factor for the growth of *L. plantarum* in green olive fermentation brine [15].

The increase in the fermentation duration observed when the initial NiA concentrations were between 0.4 mg l^{-1} and 10 mg l^{-1} was unexpected, because fer-

mentation duration usually decreases when vitamins are added. This result suggests that lactate production weakens the yeast. Although cells are stimulated to produce lactate, cell activity may decrease when large amounts of lactate are produced and only small amounts are exported out of the cells. A build-up may be toxic to the yeast and slow the metabolism. The regulation of pH improves lactate production and final sugar consumption; and thus it is important to keep the pH constant to maintain good cell activity in lactate-producing K1-LDH cells. However, pH regulation cannot prevent acidification of the cytoplasm. To gain further insight into lactate production, it would be useful to study the mechanism by which lactate, mostly dissociated in the cytoplasm, is exported. Cássio et al. [5] hypothesised that lactic acid can only freely diffuse through the membranes when it is in its undissociated form. As the cytoplasmic pH (about 6.5) is much higher in yeast cells than the lactic acid pKa value (3.78), almost all of the lactic acid produced must be in the dissociated form and must be actively transported out of the cells. The transport of lactate from medium to cytosol has been described [4, 5], but the extrusion of lactate from the cytosol is still poorly understood.

From a biotechnological standpoint, we showed that NiA is a key vitamin and a limiting factor for lactate production during the entire K1-LDH fermentation process. To identify the mechanism involved in this control, NiA concentrations have to be followed throughout the fermentation process. We obviously need to improve our understanding of the physiological processes involved. We should for instance investigate how NiA affects LDH activity. The first approach will be to study the in vitro induction of LDH activity by NiA. In vivo approaches like transcriptional profiling and metabolomics could also be considered to get better insight into cell physiology and determine the true role of NiA in lactate production.

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